A STUDY OF RADIOPHOSPHATE UPTAKE IN PARAMECIUM MULTIMICRONUCLEATUM

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It was our original intent to find out if it is possible, by the use of radioactive elements, to tag microorganisms such as Paramecium, in order that an adequate evaluation of their place in food chains could more accurately be determined. It is possible under certain conditions to render paramecia sufficiently radioactive with inorganic radiophosphate that single animals will give a definite positive count over background. Thus it should be quite possible by means of simple predation experiments to calculate the utilization of microorganisms in specified situations. Coffin *et al.* (1949) and Hayes and Coffin (1951), by adding P^{32} to small lakes, have established by this relatively simple means that phosphate distribution among members of the community was much more rapid than previously supposed and that there was an extensive exchange of elements between organisms. Phosphate turnover was greatest among the algae. Another interesting fact revealed by their results was that certain plants concentrate P^{32} up to 40,000 times the concentration in surrounding water.

It is important to know to what extent aquatic organisms concentrate ions in their bodies from their environment because of the implications in sewage and radioactive waste disposal problems.

Methods

The organisms used in these experiments were derived from the cultures used by Professor W. H. Johnson of Wabash College in his studies on the growth of *Paramecium multimicronucleatum* Powers and Mitchell in bacteria-free culture. All cultures were grown as described by Evans (1944). Stock as well as experimental cultures were fed on yeast and grown at room temperature (24–27° C.). At the end of 10 days, the population of paramecia reached a peak of about 450 individuals per ml. During the latter part of the logarithmic growth phase, the P^{32} was introduced as NaH_2PO_4 in weak acid solution. No carrier was added and no buffer was needed since the radioactive solution was diluted from one part in 250 of Osterhout's culture medium to one part in 2500 of Osterhout's. The Osterhout's was buffered with $M/20$ phosphate and adjusted to a pH of 7.0 with $M/10$ NaOH. The pH was measured electrochemically with a Beckman pH meter.

Several cultures for the experiments were grown; the amount of P^{32} added varied from 0.1 to 10.0 microcuries per ml. Organisms were withdrawn at intervals and washed as free as possible of the external radioactive phosphate either by dilution following centrifugations or by pipetting. Supernatant fluid from the last wash was always checked for radioactivity. In some cases, P^{32} was added to

unfed cultures, but in all other respects the organisms were treated as described above.

The washed organisms in counted numbers were placed in metal planchets with a minimum of fluid and desiccated; single animals were transferred with $\frac{1}{500}$ ml. of fluid, 10 animals with $\frac{1}{50}$ ml., and 100 animals with $\frac{1}{20}$ ml.

In order to determine if the washing was adequate, one-ml. samples of supernatant fluid from all of the successive washings were tested. Similar tests were carried out on solutions through which paramecia were transferred during washing with pipettes. It was found that no practical reduction in residual radioactivity could be effected after the fifth centrifugation. Even after 12 centrifugations, there remained some radioactivity in the supernatant fluid. Five washings brought the count down to about 10 counts per minute per ml. Kamen and Spiegelman (1948) noted that rapid centrifugation of Rhodospirillum caused considerable leakage of phosphate ions from the cells. This was also true but less so for Chlorella. They state that yeast gives up negligible amounts of P^{32}. Likewise, Moraczewski and Kelsey (1948) found that *Trypanosoma equiperdum* gave up phosphorus continuously especially as the activity of the animals decreased. They associated this with increased permeability as death of the cells approached. However, Labaw *et al.* (1950) state that after inorganic phosphate has been incorporated into the nucleic acid molecule in *Escherichia coli*, there is no interchange of the phosphorus either by metabolic exchange or death.

A certain amount of turnover is normal, but centrifugation seemed to enhance the loss of P^{32} from the cell. It was calculated that the amount of radioactivity in the medium carried over with the paramecia from the last wash to the planchets for desiccation was negligible.

Washing away the radioactive external medium by transferring with micropipettes was more satisfactory when only a few animals were desired, but this method was too slow for washing large numbers of organisms.

In a typical experiment, hundreds of paramecia were drawn out of a radioactive culture in one ml. of medium and deposited in a 15 ml. centrifuge tube. To this was added 13 ml. of fresh balanced salt solution (Osterhout's). The paramecia were centrifuged at 34 gravities for 10–12 seconds. This was sufficient to bring all normal paramecia to the bottom, but starved paramecia required about 300 gravities for 30 seconds. A glass plunger was inserted, one ml. of the medium removed for testing, and the supernatant poured off. Fresh balanced salt solution was again added and the process repeated as many times as was necessary.

Radioactivity was determined by means of a standard 64-scale Geiger counter with a Tracerlab mica window tube 1.9 mg./cm.^{2} in window thickness. The specimens were centered in planchets and counted at a distance of 5 cm. from the center of the tube. Each sample was counted for five minutes. Those with especially high levels of radioactivity were counted for one minute. All counts were corrected for decay.

Results

The presence of the radioactive phosphorus in the paramecia cultures did not appear to affect the course of the cultural history. The organisms remained the same size, their behavior was unmodified and the reproductive rate, though not measured exactly, appeared to parallel control cultures.

In the initial experiment, 0.5 microcurie of P^{32} per ml. was added to a culture of paramecia. The organisms were dividing at a maximum rate. The culture was one week old; there were approximately 350 paramecia per ml. Twenty-four hours after introduction of the P^{32}, paramecia were removed and prepared for radioactive determinations either singly or in groups of ten. While the single paramecia always registered a positive count over background it was low, averaging 3.8 counts per minute. The groups of 10 paramecia per dish showed an increase by a factor of 10 over the singles; this confirms the reliability of the individual counts.

Six days later, the radioactivity of the paramecia was again measured. The activity had increased slightly, being now 4.5 counts per minute per animal. Again the dishes containing 10 animals each showed a very close correlation with the 10 separates.

It is to be noted that the paramecia were feeding and possibly most of the intake of phosphate was through the food vacuole system, either in solution or in the food organisms.

Several new cultures, each containing thousands of thoroughly washed paramecia, were established. These received no food. Radiophosphorus was added and radioactive determinations were made at intervals (after 20 hours, 3 days, 5 days, 9 days and 11 days). The concentrations tested were 0.1, 0.2, 0.5, 0.8 and 1.0 microcuries per ml. Three cultures were set up at each of these concentrations.

The results of these experiments showed that paramecia which have no available food take in very little P^{32}. In fact, groups of 100 paramecia averaged only 8.3 counts per minute for an average of 0.08 counts per animal per minute. There was no difference in uptake between the weakest and greatest concentrations of radioactive substance. Time had little effect; the organisms were about as radioactive after 20 hours as at the conclusion of the experiment.

On the eleventh day, yeast was added to two of the cultures containing 1.0 microcurie of P^{32} per ml. The population soon began to increase. Twenty hours later, radioactivity counts nearly equaled those of paramecia in the initial experiment.

Inasmuch as many microorganisms, especially green forms, may concentrate inorganic ions inside the cell, it might be revealing to render old cultures of paramecia radioactive. Andresen *et al.* (1950), using C^{14}, were able to concentrate this element in *Stentor polymorphus* containing symbiotic Chlorella. The Stentor individuals finally yielded 283 counts per minute. Old cultures of paramecia become rust-colored and sometimes green due to the presence of bacteria and Chlorella which the paramecia ingest.

The old cultures chosen were three months old and the paramecia were very numerous and healthy. Four old cultures were selected; to two were added 0.1 microcurie of P^{32} per ml., and to the other two cultures were added 2.0 microcuries per ml. Four new cultures were started and run parallel with the old cultures.

The paramecia of the new and old cultures containing 0.1 microcurie of P^{32} per ml. showed a significant difference in radioactivity. Paramecia from old cultures showed consistently twice the radioactivity as those from new cultures. There was an even greater difference between paramecia taken from new and old cultures containing 2.0 microcuries of P^{32} per ml. Paramecia from the old cultures were five times as radioactive as were those from the new cultures. Apparently much

of their phosphate was acquired as food. The results also show that the greater the concentration of radioactive phosphate, the greater the amount of radioactivity in the paramecia. It should be pointed out that paramecia of old cultures are primarily non-dividing.

After absorption, is the phosphate tightly held in the cell or is it readily released? This question was partially answered by a series of tests. A few hundred radioactive paramecia were washed through 10 centrifugations. These were left in one ml. of the last wash. Five such tests were run. One tenth ml. of solution was removed at intervals, and the radioactivity tested. Table I shows the results.

TABLE I

Loss of P^{32} from Paramecium determined by radioactive measurement of 0.1 ml. samples of medium

Time in hours after washing paramecia in fresh medium	Counts per minute of 0.1 ml. of medium
0	1.5
24	64
66	95
116	154
168	167
192	170

The paramecia rapidly release the phosphate. Powers (1947), rendering *Paramecium aurelia* radioactive by feeding them radioactive *Aerobacter aerogenes*, noted a loss of more than one-half of the total phosphorus after 20 hours from the time the cells were separated from the source of radiophosphorus.

DISCUSSION

Paramecium multimicronucleatum can be made sufficiently radioactive with P^{32} to serve in predation experiments. However, in view of the fact that paramecia give up large amounts of radioactive phosphate within a few days, the duration of such experiments must be limited. Perhaps other radioactive substances will be found to remain longer or even indefinitely in the body of Paramecium. One interspecies experimental population, using radioactive paramecia as prey and *Didinium nasutum* as predator, has shown that the radioactivity of the prey is taken over almost if not entirely by the predator, and that the radioactivity of a dividing Didinium seems to be about equally distributed between the two daughter cells.

In natural populations, the amount of food present for Paramecium would be a deciding factor in uptake of P^{32}. Unfed paramecia seem unable to concentrate inorganic phosphate. Phosphorus is required for metabolism by microorganisms (Elliott and Hunter, 1951; Sullivan, 1950; Weisz, 1949), and the experiments reported herein indicate that most of the phosphate obtained by *Paramecium multimicronucleatum* is acquired in its food. Probably small amounts are absorbed directly and some adsorbed on the surface. Saprophytic and autotrophic forms seem to be able to absorb large amounts of phosphate directly.

Using Popoff's (1909) equation for determining volume of *P. caudatum*, it was calculated that paramecia which are feeding have roughly 20 times more radioactivity than the surrounding medium, while unfed paramecia have less than one-half the radioactivity. Mazia and Hirshfield (1950) measured the uptake of in-

organic P^{32} by Amoeba in the absence of food, and they found that the concentration of phosphate in the cell was greater than outside by a factor of at least 50. It is probable that, as in *P. aurelia* (Powers, 1947), the phosphate is present in *P. multimicronucleatum* as organic phosphate.

Summary

1. In a medium containing inorganic radioactive phosphorus, *Paramecium multimicronucleatum* become sufficiently radioactive for use in quantitative predation experiments.

2. It is possible to measure the P^{32} uptake of a single individual.

3. When food is absent, Paramecium does not take in inorganic phosphate in solution. The phosphate is acquired in measurable amounts in its food.

4. The phosphate taken in is rapidly lost from the animals, probably as a result of an organic turnover.

5. Paramecia in old cultures, especially cultures containing Chlorella, become more radioactive than those in other cultures probably because the Chlorella which are ingested by the paramecia, absorb much phosphate, and also because old cultures of paramecia are primarily non-dividing.

6. The greater the initial concentration of P^{32} in solution, the more radioactive the paramecia become.

7. When washed by centrifugation, there is a leakage of P^{32} from the organisms over and above the normal turnover of phosphate.

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